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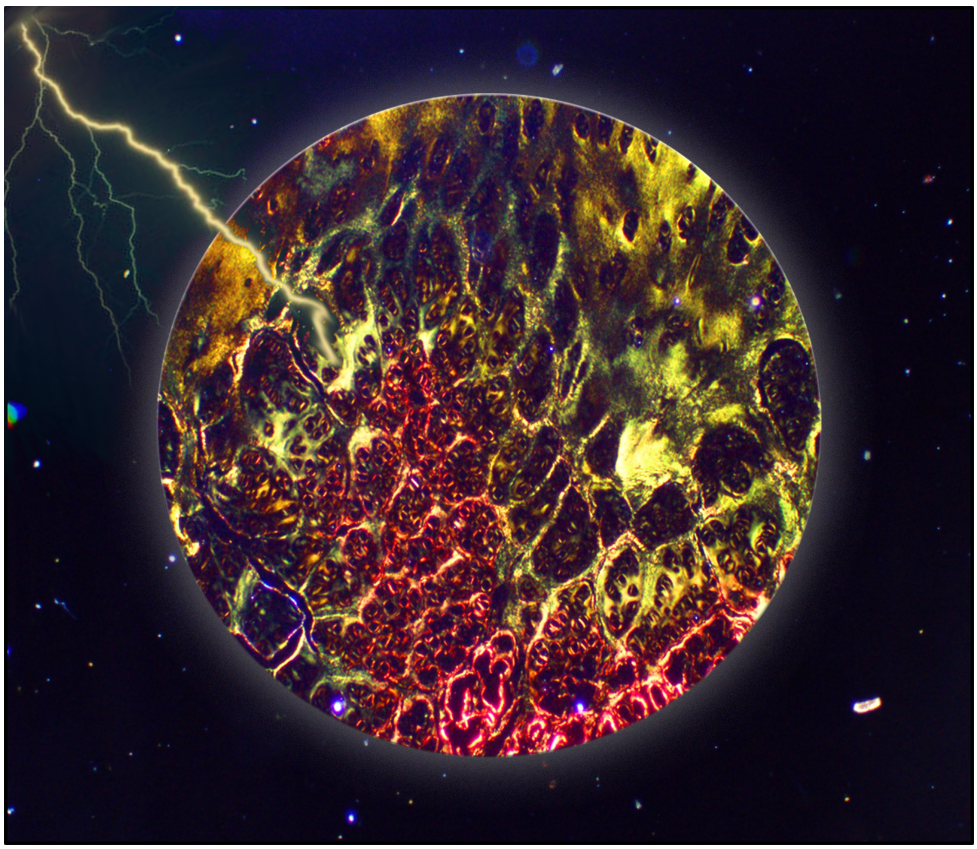
EFFECT OF SHOCK WAVE THERAPY ON LONGITUDINAL BONE GROWTH AND GROWTH PLATE CARTILAGE



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Effect of shock wave therapy on longitudinal bone growth and growth plate cartilage

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Sowmya Ramesh

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To my future self! –

I hope that the future version of myself will be glad to see this.

POPULAR SCIENCE SUMMARY OF THE THESIS

Can sound waves make your bones grow longer?

Each individual varies considerably in height, and this can be attributed to differences in the function of the growth plate, a specific structure located at each end of our long bones. The growth plate is very active until adolescence and is then gradually replaced by bone whereafter we no longer can grow. Any damage/injury to the growth plate before closure can lead to abnormal bone growth or leg length difference. Existing operating and medical ways to correct such abnormalities are effective but may have different side-effects. In this thesis project, I have studied if a type of sound waves (shock waves) can be used to stimulate growth plate cartilage from animals and humans and if bone growth can be accelerated in different animal models. In rat and rabbit bones, sound waves increased the bone length after treatment by activating different genes responsible for bone growth. When the growth plate cells were damaged, shock wave treatment was found to prevent them from dying. In human growth plate tissue, shock wave treatment was found to increase gene markers required for bone elongation. In the future, encouraging results from this study may help us find a new method to treat leg length difference, avoiding the known side-effects of existing surgical techniques.

ABSTRACT

Aim: The overall aim of this thesis was to investigate the potential for radial shock wave treatment (rSWT) to regulate growth plate chondrogenesis and longitudinal bone growth. It was based on the hypothesis that the effects are dose-dependent in a bimodal response pattern where low-energy rSWT will increase chondrocyte proliferation/hypertrophy, and thereby bone growth, while high frequency/energy rSWT will induce chondrocyte apoptosis and thereby, premature growth plate closure.

Methods: To achieve this, we experimentally studied the role of rSWT in three different model systems: *in vitro* cultures of a mouse chondrocytic cell line, in *ex vivo* organ cultures of fetal rat metatarsal bones and human growth plate cartilage, as well as *in vivo* in rabbits.

Results: A single session of high-energy rSWT was capable of locally promoting longitudinal bone growth in rat metatarsal bones cultured under normal physiological conditions. Detailed immunohistomorphometric analysis of sectioned growth plate cartilage revealed that this stimulatory effect was linked to augmented chondrocyte proliferation and hypertrophy, and decreased apoptosis. Furthermore, mechanistic studies of molecular markers governing growth plate chondrogenesis showed increased expression of PTHrP, GLI-1, NFkB, and IGF-1 in the bones exposed to rSWT **(Paper I)**. Next, we investigated the role of rSWT in an *ex vivo* model of growth impairment where Hh signaling was blocked. Cultured fetal rat metatarsal bones were challenged to two different Hh inhibitors, vismodegib and GANT61, causing growth retardation, while when combined with a session of rSWT the inhibitory effects on growth plate chondrogenesis and bone growth were partially abrogated **(Paper II)**. Thereafter, we performed *in vivo* studies in immature and adolescent rabbits and observed that high-energy rSWT increased the formation of chondrocyte columns and longitudinal bone growth, respectively **(Paper III)**. Finally, mechanistic studies revealed that rSWT caused upregulation of chondrogenic genes when studied in a unique model of cultured human growth plate cartilage (*SOX9*, *GLI-1*, *IHH*, *COL-X*, and *IGF-1*) and also in the mouse ATDC5 chondrocytic cell line (*Acan*, *Sox9* and, *Col2a1*) **(Paper IV)**.

Conclusion: Altogether, our observations, verified in preclinical and *in vitro* models, propose that high-energy radial shockwave treatment could potentially be used to stimulate growth plate chondrogenesis and longitudinal bone growth.

LIST OF SCIENTIFIC PAPERS

I. **Ramesh S**, Zaman F, Madhuri V, & Sävendahl L. (2020). Radial extracorporeal shock wave treatment promotes bone growth and chondrogenesis in cultured fetal rat metatarsal bones. Clinical orthopaedics and related research 478(3), 668–678.

II. **Ramesh S**, Sävendahl L, Madhuri V, & Zaman F. (2020). Radial shock waves prevent growth retardation caused by the clinically used drug vismodegib in *ex vivo* cultured bones. Scientific reports 10(1), 13400.

III. **Ramesh S**, Zaman F, Sävendahl L, & Madhuri V. Radial extracorporeal shock wave treatment promotes growth plate chondrogenesis and longitudinal bone growth in rabbits.
Manuscript.

IV. **Ramesh S**, Zaman F, Shaji RV, Sävendahl L, & Madhuri V. Molecular effects of radial shock wave treatment in the ATDC5 chondrogenic cell line and *ex vivo* cultured human growth plate tissue.
Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

SOC	Secondary ossification center
GH	Growth hormone
IGF	Insulin-like growth factor-1
SOX9	Sex determining region Y-box 9
PTHrP	Parathyroid hormone-related peptide
Ihh	Indian hedgehog homolog
TGF-β	Transforming growth factor beta
COL-X	Collagen alpha-1 (X) chain
RNA	Ribonucleic acid
Wnt	Wingless-type
NO	Nitric oxide
MAPK	Mitogen-activated protein kinase
ROCK	Rho-associated protein kinase
TNF-α	Tumor necrosis factor-alpha
NFκB	Nuclear factor-κappa light-chain-enhancer of activated B-cells
ESWT	Extracorporeal shock wave therapy
rSWT	Radial shock wave treatment
CP	Cerebral Palsy
BTX	Botulinum toxin
MSC	Mesenchymal stem cells
mTOR	Mammalian target of rapamycin
FAK	Focal adhesion kinase
PCNA	Proliferating cell nuclear antigen
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
GLI-1	Glioma-associated oncogene homolog 1
VEGF	Vascular endothelial growth factor
GANT61	Glioma-associated Oncogene antagonist
COL2A1	Collagen type II, alpha 1
ACAN	Aggrecan

1 INTRODUCTION

If I have a thousand ideas and only one turns out to be good, I am satisfied, said Alfred Nobel.

All scientific discoveries lead to something, and some are truly groundbreaking. I have always believed that most of the research questions that involve a basic understanding of cellular origin and function have been attempted in the yesteryears with limited technology and resources that were available then. Based on the historic leads built from the past, we are now refining our knowledge by using cutting-edge technology. The research question asked in this thesis is no exception to this statement. I would say that the groundwork for the current study was established in the 1980s.

At the beginning of this current research, shock wave technology was already an established clinical therapy in children with musculoskeletal disorders. Also, in one of my institutions, it was started as a new therapy for children with muscle spasticity. Based on the very few existing preclinical studies, shock wave treatment is deemed to be contraindicated in young growing children. It is therefore understood that it should not be used in the area around the knee and near growing physis. Therefore, before proving its efficacy on repairing spastic muscle, the effect of shock wave treatment on physeal cartilage needs to be evaluated. This observation was the foundation and motivation for us to carry out the present study and expand the knowledge to understand if shock wave therapy can be used as a potential non-invasive modality to regulate bone growth in children. During the study proposal, the hypothesis was expanded wherein a positive or negative effect on growth could be harnessed for therapeutic growth modulation while no change establishes safety. In addition to being of interest to both endocrinologists and orthopedic surgeons, another motivation and relevance for this collaborative study was that the children in India are short-statured while those in Sweden are extremely tall-statured.

In the subsequent sections, I have outlined the basics of longitudinal bone growth, the relevance of shock wave therapy in this context, how the present study complements the pioneering studies and what is the new knowledge that we have gained.

2 LITERATURE REVIEW

One of the unique aspects of longitudinal bone growth is the presence of the growth plate (or physis) that is sandwiched between the epiphyseal and metaphyseal region of a long bone. Bone growth is majorly dependent on the interaction of many hormonal, local growth factors, as well as mechanical factors. As the growth plate cartilage in children is delicate, it is more susceptible to injuries due to acute trauma. Other etiologies leading to premature growth impairment or abnormal growth include tumors, infections, and genetic mutations, or hormonal deficiencies (Nilsson, 2018; Peters, Irving, & Letts, 1992; Shapiro, 1982; Stanton & Abdel-Mota'al, 1998; Wilson & Thompson, 1939).

An altered growth followed by a deformity can also result from abnormal mechanical loading. This phenomenon also referred to as mechanical modulation of bone growth, has significant implications in the progression of various congenital musculoskeletal deformities such as genus varus/valgus, tibia vara/valga, and limb length discrepancy (Aitken, 1976; Scheffer & Peterson, 1994). Surgical management of such deformities is focused on altering the mechanical environment of deformed bones. For instance, external fixators are attached to the bone for limb lengthening, while eight plate screws are applied to the growth plate to slow down bone growth. However, there is a limited basic understanding of how mechanical forces regulate bone growth.

This review of published work and my own experience summarizes the current state of research concerning critical questions about the underlying mechanisms of biomechanical mediated bone growth. I have discussed growth plate chondrocyte mechanobiology, how it contributes to bone growth, and how mechanical stress affects the structure and function of growth plate chondrocytes.

2.1 NORMAL PHYSEAL ANATOMY

2.1.1 Gross

All long bones (clavicle, humerus, radius, ulna, femur, tibia, and fibula) have growth plate cartilage at both ends whereas smaller bones (metacarpals, metatarsals, and phalanges) have a physis at one end only. During various phases of postnatal growth and development, a secondary ossification center (SOC) appears within the epiphysis (Kronenberg, 2003). The secondary ossification center persists until the closure of the growth plate and aids to demarcate the physeal zone on the radiographs. Recent evidence suggests an evolutionary reason behind the onset of SOC, where it acts as a shielding structure to protect the weakest region of the growth plate cartilage from high mechanical stress (Xie et al., 2020). In humans, the overall growth rate decelerates until skeletal maturity at the end of the early pubertal stages, despite pubertal growth spurts during the adolescence period.

2.1.2 Microscopic

The histological architecture of the growth plate is well-organized and relevant to understand the concept of linear bone growth. Conventionally from the center of the epiphysis to the metaphysis, the growth plate cartilage is divided into four zones: resting (germinal), proliferative, hypertrophic, and calcification (Emons, Chagin, Sävendahl, Karperien, & Wit, 2011; Kirkwood & Kember, 1993). The chondrocyte proliferation is localized to the resting and proliferative zones, whereas the remaining two zones are categorized by the hypertrophic chondrocytes, matrix production, cell death, and calcification. The process by which longitudinal bone growth occurs primarily by the transformation of cartilage to the bone is termed endochondral ossification (Kember & Sissons, 1976). It contrasts with the appositional bone growth where the mesenchymal progenitor cells in the periosteum are directly converted to osteoprogenitors via intramembranous ossification (Debnath et al., 2018). The zone of Ranvier consists of the fibroblasts, chondroblasts, and osteoblasts and responsible for increasing the width of the bone (Rockwood, 2010). The perichondrial ring on LaCroix imparts mechanical stabilization of epiphysis to the metaphysis. Although the growth plate is considered avascular, the epiphysis and SOC receive their blood supply that enters through the perichondrium to remain active (Trueta & Morgan, 1960). To allow the normal process of bone development, there exists an inherent genetic switch that regulates the differential growth processes. These adaptations vary between species as the bone growth rate is connected to the number of cells distributed in each of the growth plate zones (Kirkwood & Kember, 1993) (E. B. Hunziker, 1994) (E. Hunziker, Schenk, & Cruz-Orive, 1987).

The proportion and the number of chondrocytes present within the growth plate zones vary relatively between different species (**Figure 1)**. For instance, avian and human growth plates have a more expansive resting zone (Farnum & Wilsman, 1998). Sergerie et al. (2009) has quantified the contribution of resting, proliferative and hypertrophic zones in neonatal ulnar porcine growth plates to be 70%, 17%, and 13%, respectively (Sergerie, Lacoursière, Lévesque, & Villemure, 2009). In contrast, in 3-weeks-old proximal tibiae rat growth plates, the corresponding zones were 6%, 35%, and 59% (E. Hunziker & Schenk, 1989). Since the long bone growth is directed away from the proliferative zone, the growth plates are considered monopolar. These are unlike the iliac apophyseal growth plates (bipolar) where growth occurs bidirectionally with resting chondrocytes in the middle, and proliferative and hypertrophic chondrocytes on both sides (Villemure & Stokes, 2009).

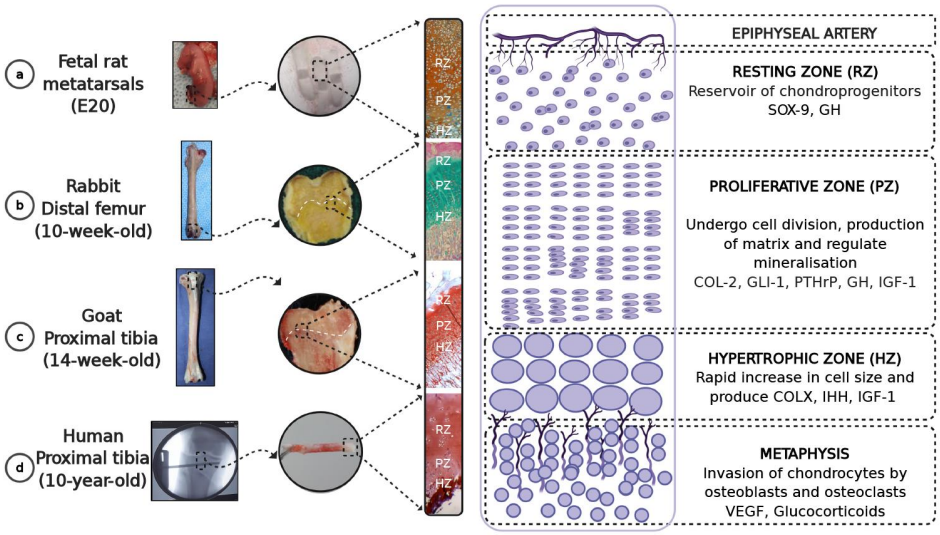

Figure 1. **Gross and differences in the histological architecture of the growth plate zones across species. a**. Fetal rat metatarsal bones; **b**. Rabbit distal femur; **c.** Goat proximal tibia; and **d.** Human proximal tibia. White interrupted lines indicate the growth plate cartilage. Note the differences in the cellularity, organization of the column, as well as the thickness in the resting zone (RZ), proliferative zone (PZ), and hypertrophic zone (HZ). A schematic picture of the growth plate architecture and key intrinsic signaling pathways in each growth plate zone (right). The illustration represents the different cellular zones; epiphyseal and metaphyseal bone on each end of the growth plate contains blood vessels that supply each region of bone.

2.2 WHAT ARE THE FACTORS INFLUENCING BONE GROWTH?

2.2.1 Hormonal regulation of growth plate cartilage

Growth hormone (GH) and insulin-like growth factors (IGFs) in the systemic circulation effectively stimulate bone growth **(Figure 2)**. A breakthrough study by Olle Isaksson more than 30 years ago found that local injection of growth hormone directly stimulates growth plate cartilage and local production of IGF-1 (Isaksson, Jansson, & Gause, 1982). Preclinical studies have shown intrauterine and postnatal growth retardation in mice lacking the *igf* (Baker, Liu, Robertson, & Efstratiadis, 1993) and GH genes (Zhou et al., 1997), respectively. An imbalance of the GH/IGF-1 axis due to other factors also influences final bone length. The presence of excess glucocorticoids leads to decreased growth rate in humans (Allen, 1996) and animals (Altman, Hochberg, & Silbermann, 1992; Chrysis, Ritzen, & Sävendahl, 2003). Thyroid hormone and sex steroids contribute to accelerated bone growth and the prepubertal growth spurt (Kindblom et al., 2001; Nilsson, Marino, De Luca, Phillip, & Baron, 2005). Estrogens directly stimulate the pituitary gland to release GH. Growth hormone is secreted in a pulsatile manner during childhood, and during puberty under estrogen stimulation, there is an increased burst of GH, and this drives IGF-1 production, primarily from the liver. Estrogens also have direct effects on the growth plate, stimulating growth plate fusion. In a male patient, Eric Smith in 1994 made a breakthrough discovery showing that a mutation in estrogen receptor alpha prevented growth plate fusion to occur highlighting the crucial role of estrogen action for this important process (Smith et al., 1994).

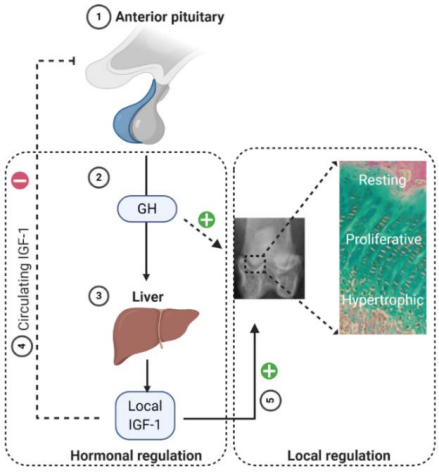

Figure 2. Hormonal regulation of growth plate cartilage. A schematic representation of the local and systemic actions of the GH/IGF-1 axis on longitudinal bone growth. The illustration represents the essential signaling pathway in which GH and IGF-1 influence longitudinal bone growth. Apart from stimulating IGF-1 production in the liver, GH also stimulates growth plate via local secretion of IGF-1 in growth plate chondrocytes. The red symbol indicates the negative feedback mechanism of IGF-1 inhibiting GH synthesis and release.

2.2.2 Local regulation of growth plate cartilage

The resting zone of the growth plate is the region adjacent to the ossifying epiphysis. With a round morphology, these cells possess a high matrix synthesis to cell volume ratio with relatively low mitotic activity (quiescent state). A transcription factor, SOX-9, is responsible for maintaining chondrocytes in their native phenotype and synthesizing its associated matrix for survival (Leung et al., 2011). It is followed by the zone of proliferation which is identified by longitudinal columnar-like appearance. As early as 1987, Brighton et al. put forward a theory that the resting chondrocytes do not contribute directly to the growth process. He hypothesized that the proliferative chondrocytes that exist closest to the resting zone are stem-cell-like and that upon cell division, the upper daughter cells may continue to be stem-cell-like while the lower daughter cell contributes to proliferation (Brighton, 1987). While later in 1994, Hunziker postulated that the resting chondrocytes might serve as the hub for stem-cell-like cells, divide only upon a stimulus and continue to repopulate upon division (E. B. Hunziker, 1994). The latter hypothesis was supported by Abad et al. in 2002 when he demonstrated that chondrocytes in the resting zone alone could replenish the proliferating and hypertrophic zone in a rabbit model (Abad et al., 2002). More recently, two studies back-to-back provided compelling evidence using the confetti mice model that indeed the resting zone houses stem-like cells (Mizuhashi et al., 2018) and are positive for stem cell marker, CD73 (Newton et al., 2019). It is indeed very intriguing to see how the mystery of growth plate biology has unraveled over the years.

In the proliferative zone, cells begin to multiply and have a flattened phenotype; the matrix is enriched with collagen type II, IX, and XI. Around nine decades ago, Dodds proposed that the columnar arrangement of the cells is driven via chondrocyte rotation. Cell division occurs by assuming a compressed phenotype along the mediolateral axis, rotating 90° and stacks on top of each other such that it is proximodistal to the axis of the long bones (Dodds, 1930). While growth plate flipping experiments in rabbits, suggest that resting cells secrete morphogens to orient them in this manner (Abad et al., 2002). More recently, it has been shown that the mechanical forces via the organization of the actin cytoskeleton (Killion, Mitchell, Duke, & Serra, 2017) and noncanonical frizzled signaling acting on the growth plate cartilage (Li & Dudley, 2009), together regulate chondrocyte polarity. Although the columnar arrangement of chondrocytes is conserved across species, there is a striking difference in their mitotic activity. One proliferating chondrocyte in a 4-week old rat takes about 48 hours to complete its cell cycle in contrast to human (5-8 years of age) chondrocytes where it spends 20 days (E. Hunziker & Schenk, 1989; E. Hunziker et al., 1987; Kember & Sissons, 1976). Every new proliferative chondrocyte that is produced contributes to an increase in growth plate height equivalent to its height, approximately 7 μm (E. B. Hunziker, 1994). This rate of chondrocyte proliferation is controlled locally by several paracrine factors such as parathyroid hormone-related peptide (PTHrP), Indian hedgehog (IHH) and transforming growth factor-beta (TGF-β) and IGF-1 (Kronenberg, 2003).

The region, at which cell division terminates and is accompanied by swelling of their lacunae is termed as the hypertrophic zone. It is marked by an increase in type X collagen (COL-X) expression and identified by the presence of blood vessels, osteoclasts, osteoid, woven bone, and osteoblasts. It is considered a significant contributor to long bone growth. A new hypertrophic chondrocyte takes only about 9 hours to contribute to an 8 μm increase in bone length and up to 20% increase in growth rate may be achieved by selectively modulating the cells in this zone (E. Hunziker & Schenk, 1989).

The overall process of endochondral ossification is believed to be mediated either by classical apoptosis where osteoblasts invade the chondrocytes, hypertrophic chondrocytes transdifferentiate into osteoblasts, hypoxia (low oxygen), or autophagy (Emons et al., 2011). Each paradigm mentioned above is still a subject of debate in the process of epiphyseal fusion. It has been shown that when an individual approaches skeletal maturity, there will be an exhaustion of resting chondrocytes and a significant reduction in cell proliferation and division (Schrier et al., 2006). Histomorphometric studies of the growth plate also support this finding where growth plate fusion is accompanied by structural changes (Alexander, 1976; Kember & Walker, 1971). Some of the essential endocrine factors that influence bone elongation are GH, IGF-1, steroid hormones (estrogen and androgen), thyroid hormone and glucocorticoids (Sävendahl, 2005; Weise et al., 2001). Thus, coordinated action of both paracrine and endocrine factors is indispensable for the regulation of linear bone growth.

2.3 CLINICAL CONDITIONS WITH GROWTH FAILURE

Technological advances in the recent past have been possible due to laser microdissection, small RNA sequencing, tissue-specific gene targeting in mice, and genetic screening of rare diseases. As we now understand that the growth plate is tightly regulated by paracrine and endocrine signaling pathways to function normally, mutations in the genes underlying these signaling cascades lead to abnormal growth and present as a skeletal deformity. Some of the clinical conditions are listed in **Table 1**.

Table 1. Clinical conditions due to impaired chondrogenesis

Defects in the early stages of chondrogenesis		
Defects in the local growth factors	Heterozygous mutations in SOX9	Campomelic dysplasia (Kwok et al., 1995)
Defects in late stages of chondrogenesis		
Defects in the local growth factors	Inactivating mutations in its receptor (PTH1R) result in Blomstrand dysplasia	Blomstrand dysplasia (Duchatelet, Ostergaard, Cortes, Lemainque, & Julier, 2004)
	Homozygous mutations in IHH gene	Acrocapitofemoral dysplasia (Hellemans et al., 2003)
	Heterozygous mutations in Runx2	Cleidocranial dysplasia (Otto, Kanegane, & Mundlos, 2002)

2.4 SURGICAL INTERVENTION FOR GROWTH MODULATION

2.4.1 Growth stimulation

Alessandro Codivilla was the first to introduce the surgical technique for bone lengthening in 1905. By applying stepwise traction, lengthening can be achieved (Codivilla, 1905). Later in 1951, Ilizarov applied the principle of distraction osteogenesis to treat a wide range of conditions such as nonunions, congenital deformities and bone shortening (Codivilla, 1905). This method uses an external fixator with two ring blocks compressed together. The bone and soft tissue are distracted at a rate of 1 mm per day divided into four parts; patients between 13 and 15 years of age and height between 110-150 cm are suitable for the procedure. More recently, computer-assisted circular frames such as the Taylor Spatial Frame have gained a lot of attention. The next step in the evolution of this technique was the use of a self-distraction motorized nail that magnetically drives the titanium intramedullary complications to overcome the complications of the external fixation (Green, 2017). However, Ilizarov is still considered as a gold standard for all limb lengthening procedures. Though the technique is effective in improving the growth, it is time-consuming and often associated with reduced range of motion in Achilles tendon post-surgery. Other common complications include pin-tract infections, poor regeneration, and axial malalignment (Paley, 1990).

2.4.2 Growth arrest

Epiphysiodesis, introduced by Phemister in 1933, is a surgical method by which growth plate cartilage is destroyed to restrict longitudinal bone growth (Phemister, 1933). The technique has evolved over the years and is considered safe in significant growth reduction (Benyi et al., 2010; Canale, Russell, & Holcomb, 1985). A reduction in height by about 4-7 cm after bilateral percutaneous epiphysiodesis has been reported (Benyi et al., 2010; Odink et al., 2006). Also, for the treatment to be effective, the recommended age for surgery is before bone age 12.5 years in girls and 14 years in boys (Benyi et al., 2010). Reversible methods of epiphysiodesis include physeal stapling, guided growth plates (8 plate, peanut plate of Biomet). The risk of infection and the possibility of skeletal deformities, including leg length differences are, however, the downsides of any surgical procedure (Terrill & Dunn, 2014).

2.5 NON-SURGICAL INTERVENTION FOR GROWTH MODULATION

2.5.1 Growth stimulation

Human growth hormone has been used in the treatment of GH-deficient children for more than 3 decades (Gharib et al., 2003; P. A. Lee et al., 2012). It aims at promoting linear growth and stopped after the fusion of the physis (Novikov, Subramanyam, Muradisinov, Novikova, & Kolesnikova). Though rGH therapy improves linear bone growth, the safety of the treatment has always been a concern. A recent study has evaluated the long-term safety (15 years) in GH-treated patients. The outcome suggests that there is no relationship between GH dose and the occurrence of adverse events in patients receiving rGH therapy (Sävendahl et al., 2020; Sävendahl, Pournara, Pedersen, & Blankenstein, 2016). Another concern is the risk of cancer associated with hormonal therapy. This was addressed by the SAGhE European cohort study where no relation between the duration or dose of GH therapy and the risk of cancer was observed. However, those patients treated after previous cancer had an increased risk of cancer with increasing GH dose (Swerdlow et al., 2017). These data suggest further long-term follow-up in patients with no history of malignancy, as the manifestation is more common in the older population (e.g. prostate cancer).

2.5.2 Growth arrest

Non-surgical treatment for height reduction includes sex steroid therapy which has been in practice since 1956 for girls (Goldzieher, 1956) and 1998 for boys (Drop, De Waal, & de Muinck Keizer-Schrama, 1998). Though considered to be effective in reducing growth (de Waal, 1996), safety is still a matter of concern. In contrast to the testosterone treatment, estrogen therapy in girls is associated with a reduction in fertility as a long-term side effect (Hendriks et al., 2011; Venn et al., 2008). Also, reports suggest an increased incidence of melanoma with high-dose estrogen treatment (Benyi et al., 2014). Some of the other hormonal therapies include somatostatin (Hindmarsh, Pringle, Stanhope, & Brook, 1995) and bromocriptine (Brion, Murrieta, & Job, 1985) with preliminary reports on efficacy and safety.

2.6 BIOMECHANICAL STIMULATION OF BONE AND GROWTH PLATE CARTILAGE

Bones undergo continuous remodeling depending on the adjacent mechanical microenvironment. A German surgeon Julius Wolff in 1893 put forth a law that a bone tissue subjected to mechanical lading will regenerate and those not subjected to stress will atrophy. This states that the bone density will depend on its stress conditions. While the biomechanical influence on linear bone growth is regulated by Heuter-Volkmann law where increased mechanical compression results in growth retardation while reducing loading accelerates growth (Villemure & Stokes, 2009).

2.6.1 Mechanotherapy at the molecular level

Chondrocytes and osteocytes respond well to mechanical cues. Several cell-signaling pathways have been identified as targets in response to mechanical stimuli. Some of the key players in bone repair and formation include intracellular calcium ion signaling, prostaglandin and nitric oxide (NO) signaling, and Wingless-type (Wnt)/β-catenin signaling (Huang & Ogawa, 2010). Integrin signaling, TGF-β/Smad, mitogen-activated protein kinase (MAPK) signaling, Rho-associated protein kinase (ROCK) signaling, tumor necrosis factor-α (TNF-α)/nuclear factor-kappa light-chain-enhancer of activated B cells (NF-κB) signaling, and Wnt/β-catenin pathways are involved in treating disorders with abnormal fibroblast proliferation (Huang & Ogawa, 2012). By selectively targeting these signaling molecules using specific inhibitors, it may be possible to reverse the disease phenotype of the cells to treat a disease.

At the most superficial level, mechanotherapy alter the signal transduction by controlling one of the following stages: (i) mechanocoupling phase, where the exerted mechanical signal perceived by the mechanosensors of the cell; ii) biochemical coupling, where the mechanical signal is translated into a biological signal, a language that is understood by the cells eventually leading to changes in transcription factors and protein expression; (iii) signal transmission, where the biological signal from the receiver cell is transferred to the effector cells leading to activation of signals in different cell types; (iv) cellular response, where there is an overall change in the gene expression pattern **(Figure 3)** (Huang, Holfeld, Schaden, Orgill, & Ogawa, 2013; F.-Y. Lee et al., 2017; Turner & Pavalko, 1998; Villemure & Stokes, 2009).

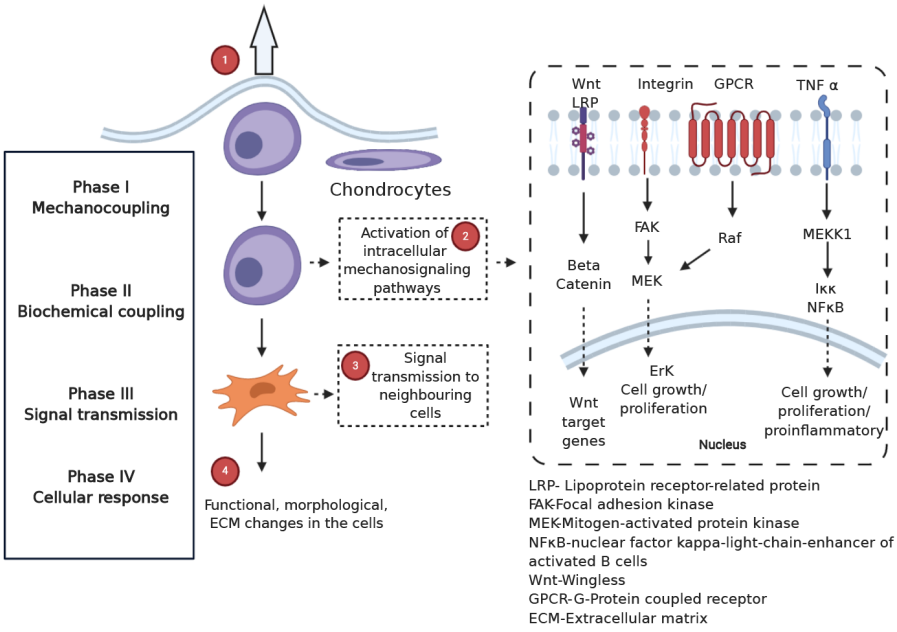

Figure 3. **Phases of mechanotransduction following mechanical stimulus: 1.** Mechanocoupling, **2.** Signal transduction, **3.** Activation of neighboring cells and **4.** Matrix synthesis. *Source: Adapted and modified from (Huang et al., 2013).*

2.6.2 Mechanotherapy at the cellular level

Some of the neurodegenerative and growth plate defects conditions result in cellular dysfunction by affecting the cytoskeleton (McGlashan, Haycraft, Jensen, Yoder, & Poole, 2007; McMurray, 2000). Integrin-mediated cellular function that affects cytoskeletal components is of great interest. Other mechanosensors including cell junctions, connexins, primary cilia, and sensory ion channels, are important. Specific fundamental changes in mechanotransduction include changes in the extracellular matrix proteins, stretch-activated ion channels, glycocalyx, cell-cell receptors, cytoskeletal components, nucleus, cytoplasm and mitochondria.

2.6.3 Mechanotherapy at the tissue level

Mechanotherapies targeting the tissue level are mainly focused on improving wound healing, and these include micro-deformational wound therapy, extracorporeal shockwave therapy (ESWT), soft tissue expansion, callus distraction, and surgical tension reduction (Huang et al., 2013). The other non-invasive mechanotherapy strategies for growth modulation such as radiofrequency ablation, mechanical loading, ultrasound, and low-level laser therapy are under different phases of preclinical investigations with minimal effect on growth. The subsequent section will only focus on shock wave therapy as it is more relevant to the topic of discussion.

2.7 SHOCK WAVE TECHNOLOGY

Mechanotransduction is a cellular process of converting mechanical stimuli into a biological response. Shock wave treatment exerts a mechanical force on the cellular components. The transient changes include alteration in the cytoskeleton, cellular adhesion and cell migration while long-term effects are mediated through regulation of different signaling pathways and molecular processes (d'Agostino, Craig, Tibalt, & Respizzi, 2015).

A shock wave is a short acoustic wave possessing energy and travelling in a three-dimensional space (Ogden, 2006). It is generated when the speed of the source exceeds the speed of the sound (e.g. a lightning strike). These waves are characterized by a discontinuous pattern in the medium they propagate; associated with a rapid increase in pressure and temperature. The strength of a shock wave is inversely proportional to the distance from the source. It is represented as a single positive pressure pulse, followed by a small negative pulse. The frequency varies from a few kilohertz to 10 MHz Unlike ultrasound, the shock wave pattern differs in having a high-pressure amplitude and single pulse. This concept was modified and adapted in the field of medicine. They can be either focused or radial waves. The differences between the two patterns are based on its wave characteristics are given in **Table 2** (Maier et al., 2002).

Table 2. Physical characteristics of shock waves generated by different mechanisms.

Characteristics	**Shock wave generators: Electrohydraulic, Electromagnetic, and Piezoelectric**	**Pneumatic ballistic (Radial ESWT)**
Pressure	200-800 bar (20-80 MPa)	4-5 bar (0.4-0.5 MPa)
Pressure rise time	0.01 μs	50 μs
Speed of shock wave	1500 m/s	20 m/s
Duration of positive pulse	<1 μs	200 μs
Energy flux density	High energy 0.27-0.60 mJ/mm^2 Low energy 0.08-0.23 mJ/mm^2	0.17 mJ/mm^2
Penetration depth	80-140 mm	30-35 mm

2.8 SHOCK WAVE THERAPY FOR MUSCULOSKELETAL DISORDER: A SERENDIPITOUS EVENT?

Extracorporeal shock wave therapy (ESWT) was first used in 1980 for kidney stone disintegration (Chaussy, 1988). In the late 1990s, Haupt was the pioneer in reporting the effect of shock waves on the bone (Haupt, 1997). Subsequently, many clinical orthopedic conditions have been treated, including Achilles tendinopathy (Furia, 2006), lateral epicondylitis of the elbow (Ko, Chen, & Chen, 2001) and calcifying tendinitis of the shoulder (Loew, Daecke, Kusnierczak, Rahmanzadeh, & Ewerbeck, 1999). However, a shock wave machine exclusively designed for orthopedic use was first introduced in 1993. It is now considered as a novel therapeutic modality as it eliminates surgical risks and pain. Since then, it has led to the treatment of various musculoskeletal disorders (Haupt, 1997). The technology was further refined by the year 2000 when radial shock wave treatment (rSWT) was introduced exclusively for superficial musculoskeletal conditions (Fig.1). More specifically, rSWT is effectively used in many centers to treat spastic muscles in children with cerebral palsy (Gonkova, Ilieva, Ferriero, & Chavdarov, 2013). Though recent studies have shown the ESWT treatment to be effective and safe in Osgood Schlatter or Sever's disease (Lohrer & Nauck, 2015; Lohrer, Nauck, Schöll, Zwerver, & Malliaropoulos, 2012), it is essential to note that ESWT must be used with caution if applied close to the growth plate and therefore is still not recommended in the pediatric population.

2.8.1 Clinical evidence for treating muscle spasticity

Cerebral palsy (CP) is a manifestation of a damaged motor neuron during brain development. Children with spastic CP have passive muscle stiffness (high collagen content) which in turn leads to muscle atrophy, muscle weakness and shortening. Botulinum toxin (BTX), passive stretching, serial plastering, and splints are the conventional treatment of spasticity (Hoare et al., 2010; Tedroff, Löwing, Haglund-Åkerlind, Gutierrez-Farewik, & Forssberg, 2010). Though BTX is effective in treating muscle spasms, it still has local side effects such as swelling at the injection site, common cold, etc. As a non-invasive option, radial shock wave treatment has been shown to produce a long-lasting reduction in spasticity without any side effects. A randomized, placebo-controlled trial, showed that the therapeutic effect on severe spastic upper- and lower-limbs lasted up to 2 months post-treatment within three sessions of ESWT (2,000 impulses, 0.10 mJ/mm^2) (Vidal, Morral, Costa, & Tur, 2011), while other studies applying a single session of ESWT on plantar flexor muscle was effective up to 4 weeks (Amelio & Manganotti, 2010) (Gonkova et al., 2013). The mechanism by which ESWT decreases muscle spasticity could be by increasing the blood flow to muscles and unlinking the functional bonds between actin and myosin (Mirea, Onose, Padure, & Rosulescu, 2014). The secondary effect of ESWT on growing children still needs to be verified.

2.8.2 Clinical evidence for non-union fractures

Focal ESWT is increasingly considered as the first choice of treatment for non-union fractures less than 5 mm in most of the European centers (especially in Germany). **Table 3** summarizes the clinical dose for each long bone (Xu et al., 2009).

Table 3. Optimized dose for different bones (Xu et al., 2009)

Site of treatment	Impulses	Energy
Lower limbs	6,000-10,000	0.62 mJ/mm^2
Humerus	4,000	0.56 mJ/mm^2
Radius and ulna	3,000	0.56 mJ/mm^2

A systematic review published in 2009 including ten studies concluded that an overall union rate after ESWT was around 72%; a larger randomized trial is necessary to better understand the clinical efficacy of ESWT on fracture healing (Petrisor, Lisson, & Sprague, 2009). While another recently published review (106 studies) suggests that 2000 impulses and an energy level that could be tolerated by the patient (three sessions at an interval of one week) are considered safe (Schmitz et al., 2015). Studies have also shown ESWT to be effective in alleviating pain and hip function in patients with early osteonecrosis of the femoral head, mediated through stimulation of angiogenic, osteogenic and anti-inflammatory factors (C.-J. Wang, Yang, & Huang, 2011).

2.9 EFFECT OF SHOCK-WAVE TREATMENT ON ARTICULAR CARTILAGE AND BONE (ANIMAL STUDIES)

A study in immature rabbits by Vaterlein et al. reported that application of shock waves at 2000 impulses, 1.2 mJ/mm^2 (Osteostar, Siemens) on the lateral femoral condyle did not induce any pathological changes or damage to the joint cartilage after 24 weeks (Väterlein, Lüssenhop, Hahn, Delling, & Meiss, 2000). Wang et al. studied the effect of low energy shock waves (200 impulses, 0.095 mJ/mm^2, DolorClast Master) on 36 rabbits with a full-thickness cartilage defect on the medial femoral condyle. Twelve weeks later, a progressive regeneration of hyaline-like cartilage in the shock wave treated group was observed (Q. Wang et al., 2011). In another study, 36 rabbits were used to determine the effect of shock waves on healing at the tendon-bone interface; the healing rate was accelerated in the shock wave treated group. Some studies have also found rSWT to induce *in vivo* bone formation in rabbits after six weeks with 4000 impulses at 0.16 mJ/mm^2 (2 sessions) (Gollwitzer et al., 2013). Attempts have also been made to understand the impact of shock waves on normal rabbit femurs where 1500 impulses, 0.5 mJ/mm^2, and 0.9 mJ/mm^2 led to damaging side effects (periosteal reaction) ten days post-treatment (Maier et al., 2002).

2.10 EFFECTS OF SHOCK WAVE TREATMENT ON GROWTH PLATE CARTILAGE

As mentioned earlier, shock wave treatment in growing children has been a subject of interest and debate. After a study by Yeaman et al., where shortening of rat tibia at ten weeks after treatment (lithotripsy) was observed, the application of ESWT was regarded as contraindicated on open growth plates (Yeaman, Jerome, & McCullough, 1989). It is a well-established fact that animal studies do not necessarily reflect what happens in humans, especially while using rats as a model. The epiphyseal fusion differs between the two as the growth plates of the rat remain open throughout life in contrast to humans where it undergoes fusion during late adolescence. **Table 4** summarizes the list of published studies so far discussing the effect of shock waves on growth plate cartilage. Of the eight studies, five-show no effect on growth plate cartilage and one study shows increased epiphyseal thickness; of the other two, one shows 1% increase in bone length while the other shows an average of 3 mm and 2.4 mm shortening of the tibia and femur, respectively. Notably, five of the studies have used a lithotripsy device and not the ESWT machine, which is specific for the orthopedic application. Altogether, it is still unclear if ESWT may affect growth plate cartilage and longitudinal bone growth.

The outcome may vary depending on the type of shock wave machine used, the amount of energy/impulses/frequency at which the waves are delivered, the target growth plate and the animal model used. No extensive study has been performed to date to delineate the effect of such independent parameters on growth plate cartilage and longitudinal bone growth.

Table 4. List of published studies so far highlighting the effects of shock wave therapy on the growth plate in different animal models

Animal model, dose	Site	Outcome
Lithotripsy ESWT		
Sprague Dawley rats (n=15); 1500 impulses, 20kV	Proximal tibia	No significant difference in growth plate thickness, 33% (2/6) showed limb shortening (not significant) at 10 weeks post-treatment. (Yeaman et al., 1989)
New Zealand white rabbits (n=6); 1000 impulses, 18kV	Distal femur	No effect on the growth plate and bone growth at 6 months post-treatment. (Van Arsdalen, Kurzweil, Smith, & Levin, 1991)
New Zealand white rabbits (n=30); 1000 or 4000 impulses, 14/21/28 kV	Proximal tibia	No effect on growth plate at 6 weeks post ESWT. (Giusti, Penteado, Santos, Alves, & Faloppa, 2005)
New Zealand white rabbits (n=18); 800 impulses, 0.32 mJ/mm^2	Proximal tibia	No effect on growth plate at 72 hours, 2 weeks, and 4 weeks post treatment. (Nassenstein, Nassenstein, & Schleberger, 2004)
New Zealand white rabbits (n=20); 1500/3000 impulses, 0.6 mJ/ mm^2 (3 sessions, 1-week interval)	Proximal tibia	Both showed an increase in growth plate thickness; no change in bone length at 6 weeks post-treatment. (Ozturk, Bulut, Oztemur, Kaloglu, & Kol, 2008)
Electromagnetic ESWT		
New Zealand white rabbits (n=18); 2000/4000 impulses, 0.08 mJ/mm^2	Proximal tibia	Valgus deformity (80%), no damage to physis and bone length at 3, 6 and 12 weeks post-treatment. (Lüssenhop, Seemann, Hahn, & Meiss, 1998)
Piezoelectric ESWT		
New Zealand white rabbits (n=14); 1000/5000 impulses, 100MPa at 1 Hz	Femoral diaphysis	1% increase in bone length compared to control at 6 weeks post-treatment. (Saisu et al., 2004)
Radial ESWT		
Wistar albino rats (n=16); 1500/3000 impulses, 0.38 mJ/mm^2, 1 Hz	Knee	At 8 months, no difference in bone length- Femur, tibia (Oztemur et al., 2013)

2.11 CELLULAR MECHANISMS INVOLVED IN TRANSDUCTION OF BIOPHYSICAL STIMULI

Several studies have looked at the effects of ESWT at the cellular level to understand the process of mechanotransduction. The most widely used cell type is endothelial cells, and results suggest increased vascularity and nitric oxide production after treatment (Rohringer et al., 2014). Raabe et al. reported a positive outcome of ESWT on cell viability, proliferation and differentiation potential of adipose-derived mesenchymal stem cells (MSCs) derived from an equine model (Raabe et al., 2013). Shock wave treatment of human umbilical cord-derived MSCs (0.16/0.24/0.42 mJ/mm^2 at 200 impulses for 12 days) increased osteogenesis by stimulating superoxide production both *in vitro* and *in vivo* (F. S. Wang et al., 2004). Other proposed mechanisms of osteogenesis mediated by ESWT include p38 MAPK pathway and activation of purinoreceptor 7 receptors (Sun et al., 2013). Another study demonstrated shock waves affect the mammalian target of rapamycin-focal adhesion kinase (mTOR-FAK) pathway in bone marrow MSCs (F.-Y. Lee et al., 2017). In a study using a human bone marrow MSC cell line, varied numbers of impulses ranging from 250 to 1000 at 0.16 mJ/mm^2 were applied. At 500 impulses, the highest cell proliferation rate was observed with the induction of Ras and TGF-β after one and twenty-four hours of treatment. An upregulation of alkaline phosphatase and collagen I was shown by day six and osteocalcin by day 12 (F.-S. Wang et al., 2001).

So far, studies have used varying energy flux density from 0.05-0.15 mJ per mm^2 and impulses ranging from 100-1000 but with no consensus as to which energy level has the maximum biological effect. There is also little evidence as to how varying frequency could affect cellular signaling. Most of the studies have applied pulse frequencies between 2 and 5 Hz. This strongly suggests that the outcome depends on the modality of shock wave treatment, number of impulses, energy level, and frequency used.

In contrast to focal ESWT, studies using rSWT at 500 impulses and 0.05 mJ/mm^2 inhibited osteoblastogenesis in osteoblasts. They reported a reduction in collagen type 1, osteocalcin and NFκB ligand at 24 hours and concluded that rSWT is not useful in bone healing (Notarnicola et al., 2012). Tanja et al. 2013 for the first time showed differential effects of radial shock waves (100, 200, 500 or 5000 impulses, 2.5 bar, 1 Hz) when applied *in vitro*. They tested the dose-dependent effect on human foreskin fibroblasts and choriocarcinoma that are of mesenchymal and epithelial origin, respectively (Hochstrasser, Frank, & Schmitz, 2016). Interestingly, cell proliferation remained unaffected in choriocarcinoma cells, while foreskin fibroblasts showed a dose-dependent increase in growth potential (Hochstrasser et al., 2016). Thus, studies using rSWT show that it induces mechanical damage to the cellular membrane rather than programmed cell death which happens during the initial exposure that is 1-2 hours following treatment.

Extracorporeal shock wave therapy has also been studied on normal fibroblasts to assess its suitability in hastening soft tissue repairs (Frairia & Berta, 2011). Other differentiated cells, such as human-derived tenocytes from ruptured tendons, show enhanced cell proliferation and migration when exposed to shock waves at 1000 impulses and 0.14 mJ/mm^2 (Leone et al., 2012). Apart from the regenerative effect, it is also useful in transfecting site-specific or systemic delivery of genes (Ha et al., 2015).

Overall, shock wave treatment triggers a biological response (mechanotransduction) which initiates the activation of specific cells such as pericytes, osteoblasts, fibroblasts, and MSCs to repair the bone or regenerate the damaged tissues. However, the exact mechanism of shock wave therapy that brings about a physiological effect needs to be further elucidated.

2.12 LACUNAE IN THE RESEARCH (GAP-IN-KNOWLEDGE)

Though existing strategies for treating pediatric growth disorders often are effective, the treatment is expensive, and long-term safety concerns exist. An alternative, effective non-invasive approach with fewer potential side-effects is therefore highly desired. Non-invasive strategies such as extracorporeal shock wave therapy, ultrasound, radiofrequency ablation, and low-level laser therapy have been explored for their potential use to modulate bone growth. Based on the observed clinical effect on fracture nonunions, we hypothesized that shock wave treatment could be used for growth modulation. An optimized shock wave protocol will allow either stimulation or inhibition of bone growth in children with extremely short or tall stature, respectively; or to be applied unilaterally to correct leg length differences. Alternatively, a dose that does not affect growth could be used for treating non-skeletal disorders. Though many studies have been conducted, the literature still lacks in understanding the overall impact of shock wave treatment on linear bone growth. The results must be interpreted carefully based on the animal model before claiming clinical relevance of the treatment. Very few studies have reported the independent effect of varying impulses, energy level, and frequency *in vitro* while the therapeutic effect in a clinical setting is still unknown. Moreover, the long-term effect of ESWT, either focal or radial, on a growing physis remains unelucidated.

Based on the existing literature, this thesis aimed to gain insights into the physiological mechanisms adopted by chondrocytes for modulation of linear bone growth following shock wave treatment and to determine how these specific activities are accommodated during a growth impaired condition.

3 RESEARCH AIMS

The overall main aim of the study was to investigate the effects of radial shock wave treatment on growth plate cartilage and longitudinal bone growth.

More specifically, I aimed to:

- Explore *in vitro* effects of shock wave treatment on growth plate chondrogenesis.
- Study if shock wave treatment can rescue bone growth in an *ex vivo* model of impaired bone growth.
- Assess the *in vivo* effects of shock wave treatment on bone growth and growth plate cartilage.
- Study the effects of shock wave treatment in *ex vivo* cultured human growth plate cartilage.

Table 5. Models used to accomplish the aims of this thesis

Model	**Species**	**Age**	**To investigate the effect of radial shock wave treatment on**
Organ culture *Ex vivo* cultured metatarsal bones	Sprague Dawley rats	Fetal day 20 of gestation	Local bone growth under normal and diseased conditions
In vivo	New Zealand white rabbits	6 weeks, 22 weeks	Bone growth under systemic conditions
In vitro cultured ATDC5 cells	Mouse	Chondroprogenitors	Temporal regulation of chondrogenesis
Organ culture *Ex vivo* cultured growth plate cartilage	Human (children)	10-15 years	Spatial regulation of chondrogenesis

4 METHODOLOGICAL CONSIDERATIONS

4.1 ETHICAL CONSIDERATIONS

In this thesis, I investigated the effect of shock wave therapy, a non-invasive technique for modulation of longitudinal bone growth. To address whether my scientific question can add value to the existing research, it is indispensable to test the hypothesis using appropriate preclinical models. To this end, three different model systems were utilized: local and rescuing effects of radial shock wave treatment were tested in an *ex vivo* model of cultured fetal rat metatarsal bones **(Paper I, II)**; systemic effects of radial shock wave treatment were studied *in vivo* using a rabbit model **(Paper III),** and local mechanistic effects were verified using *ex vivo* cultured human growth plate cartilage and mouse ATDC5 chondroprogenitors **(Paper IV)**. Here, I intend to discuss the ethical considerations involved in my research to understand how the experiments conducted using animal and human samples will add value to society.

Before I proceeded for *in vivo* testing, I attempted to address if my scientific purpose of the research is significant enough to justify the use of biological material. Furthermore, I generated proof-of-principle data using a fetal rat metatarsal organ culture **(Paper I, II)**. The institutional review board approved this study (IRB Min. no. 8513) and institutional animal ethics committee (IAEC 25/2013; IAEC 10/2019) at Christian Medical College, Vellore, India and Karolinska Institutet: Regional animal ethics committee (Permit no. 13572-2018). I considered this model, keeping in mind the 3Rs (replace, reduce and refine) and I could collect many bones from the same animal allowing many experimental groups with a minimum number of animals sacrificed. This model is ideal for demonstrating an apparent change in the bone length during the treatment and performing subsequent histomorphometric studies. I also understood the importance of having a good experimental design with precise statistical analysis to reduce the number of animals in research. The data obtained from this model should be extrapolated with caution as there are several limitations built-in to the model system used, such as lack of vascularity and systemic factors. Furthermore, the results need to be validated in a species where growth plates undergo fusion during or after sexual maturation, mimicking the normal pattern of growth plate maturation seen in humans.

Based on the *ex vivo* data, I considered performing *in vivo* experiments in immature rabbits (project 2) considering the similarity of the growth plate cartilage with that in a growing child. The institutional review board approved the study (Min. no. 8963) and the institutional animal ethics committee (IAEC 5/2014) at Christian Medical College, Vellore, India. I ensured that the animals were handled in a humane manner and caged individually with an adequate supply of water and feed; during the shock wave treatment, they were administered with appropriate anesthetics and analgesics. Before conducting a long-term *in vivo* experiment, I conducted a pilot study to verify the short and long-term effects on histology and final bone length, respectively. I understood that unlike rodents, the intraspecies variability in rabbits is much higher. Based on power analysis, I used 12 animals for my long-term experiments.

The use of human tissue samples in research is debatable. Though we conduct preclinical studies, there is a physiological difference between animals and humans, especially in the regulation of growth plate chondrogenesis. This is one of the reasons why experiments that respond well in preclinical studies do not necessarily translate when tested in patients. To bridge this gap and understand the clinical significance of this project, I obtained growth plate cartilage biopsies from children undergoing epiphysiodesis, a procedure to stop bone growth. The tissue that I obtained was removed as a part of their elective surgery without any additional procedure being performed. The study was approved by the institutional review board (Min. no. 11383) at Christian Medical College, Vellore, India. The patients' autonomy was respected by obtaining informed consent from parents and child's assent by the surgeon before the tissue harvest. The identity of the patients was not disclosed to any of the researchers handling the tissues in the lab. Patients and parents were explained that the current research using human growth plate samples would not have any immediate benefits for them, but the findings may lead to the development of a new non-invasive treatment for future patients with extreme short stature, tall stature or limb length discrepancy. I believe that we perform research using animal and human samples in a hope to answer the questions posed, and the outcome will eventually be of clinical significance.

4.2 *EX VIVO* CULTURE OF FETAL RAT METATARSAL BONES (PAPER I, II)

Pregnant rats at the 19/20th day of gestation were euthanized, and middle-three metatarsals from the hind limbs of the fetuses were harvested under a dissection microscope. The bones were pooled and collected in Dulbecco's Modified Eagle's Medium (DMEM/F12) with gentamicin. The bones were cultured in complete medium containing 50 µg/mL ascorbic acid, 1 mM β-glycerophosphate, 0.2% BSA, and 20 µg/mL gentamicin in a 5% CO_2 incubator **(Figure 4A)**. The pictures of bones in culture were taken, and the culture medium was replenished every two days. The day of dissection was identified as day 0 in culture **(Paper I)**.

To mimic a transient and permanent growth impairment **(Paper II)**, metatarsal bones were exposed to GANT61 (St. Louis, Missouri, United States; 10 µM) and vismodegib (Selleckchem, Houston, Texas, USA; 100 nM), respectively **(Figure 4B)**.

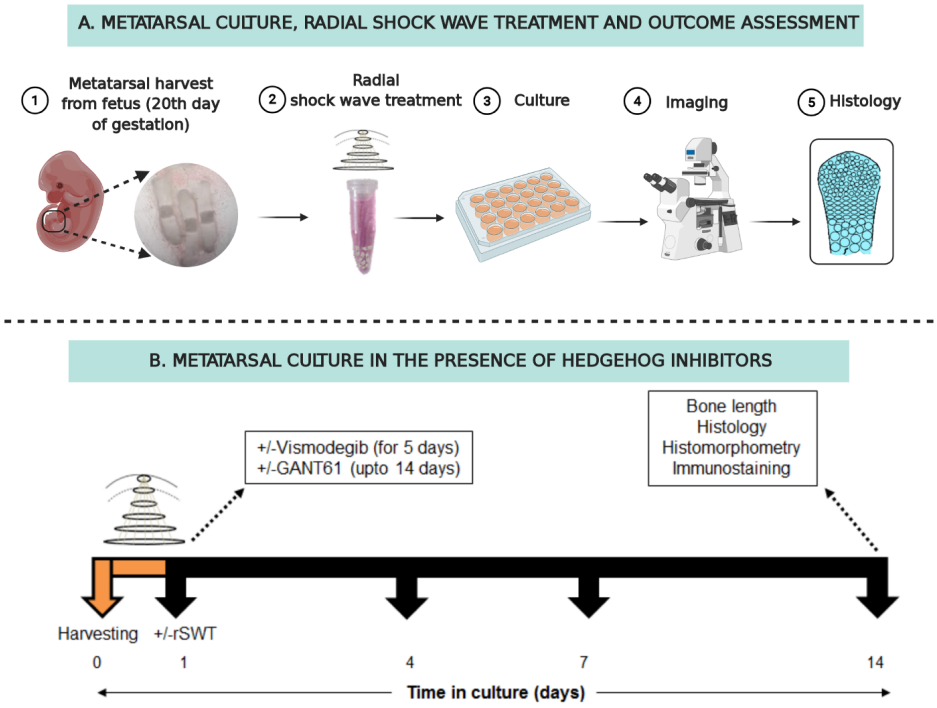

Figure 4. Schematic illustration of the experimental setting for cultured fetal rat metatarsal bones exposed to rSWT under A. Normal physiological condition or **B.** Growth impaired condition. *Source: Adapted and modified from (Ramesh, Zaman, Madhuri, & Sävendahl, 2020)*

4.2.1 Radial shock wave treatment

All experiments in this thesis were carried out using Radial Spec., Medi Spec., (Gaithersburg, MD. USA). The penetration depth of the radial pressure waves is 2 cm from the superficial surface. The 15-mm applicator probe was placed on top of the tube with ultrasound gel to avoid scattering of the waves. The radial shock wave machine used in the study is shown in **Figure 5**. For radial shock wave treatment, the bones were placed in a 1.5 ml Eppendorf tube with a complete medium. After exposure to the respective rSWT session, the bones were transferred back into a 24-well culture plate and followed for 14-15 days. The high-energy rSWT used in this experiment was 500 impulses, 10 Hz, 180 mJ.

Figure 5. A radial shock wave machine from Medispec, Gaithersburg, USA

4.2.2 Bone length measurements

The longitudinal bone growth was measured by using an inbuilt measurement tool in Leica microsystem software on day 0, 2, 4, 7, 9, 12, and 14, 15.

4.2.3 Histology, histomorphometry and immunostaining

Five-micrometer longitudinal sections of the bones were stained with Alcian blue and Safranin-O for visualizing the cartilaginous structure. Based on the cell morphology, the height of the resting+ proliferative zone and the hypertrophic zone were performed. The hypertrophic cell size was measured after COL-X immunostaining.

4.3 *IN VIVO* STUDIES IN A RABBIT MODEL (PAPER III)

The temporal effects of rSWT were investigated in healthy immature, and adolescent rabbits **(Figure 6)**.

4.3.1 Pilot study (histological study)

Six-week-old New Zealand white rabbits weighing 0.9-1 kg were divided into two groups (n=4 each). All animals were anaesthetized given an intramuscular injection of ketamine (22 mg/kg) and xylazine (2.5 mg/kg) cocktail before treatment. The right hind limb of the animal received the shock wave treatment while the left hind limb was untreated and acted as a control. Each animal received a low-energy dose of 1500 impulses, 5Hz, 90 mJ and a high-energy dose of 3000 impulses, 10 Hz, 180 mJ. The treatment was administered four times (at one-week intervals). After six weeks of follow up, all animals were sacrificed by intraperitoneal Pentothal Sodium injection (5 mg/kg).

4.3.2 Longitudinal bone growth study

A total of twelve 22-week-old rabbits were treated with radial shock waves (3000 impulses, 5 Hz, 180 mJ) four times at an interval of 1-week. The animals were further followed up for seven weeks after the last treatment. Bone length measurements were performed on the harvested tibia at the end of the sacrifice.

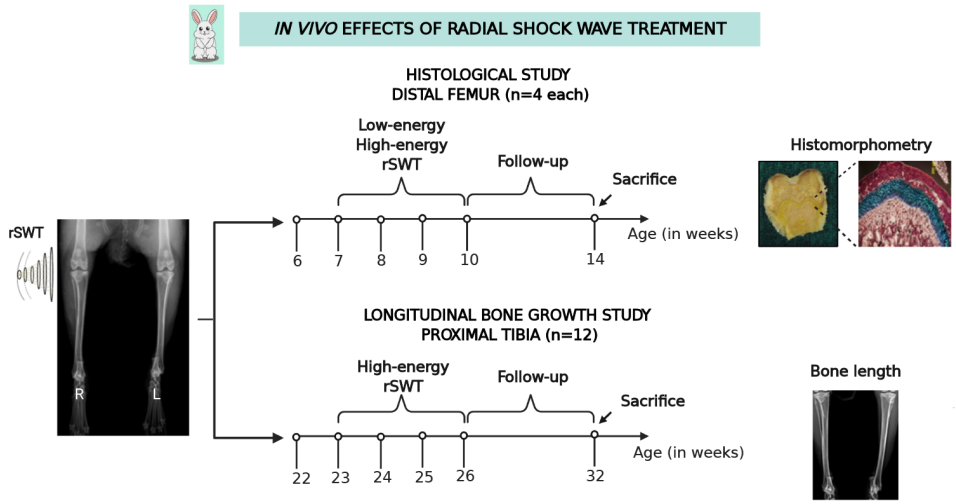

Figure 6. Experimental overview of *in vivo* rabbit studies. A histological pilot study was conducted in immature rabbits, and a longitudinal bone growth study was performed in adolescent rabbits.

4.4 *EX VIVO* CULTURE OF HUMAN GROWTH PLATE CARTILAGE (PAPER IV)

To study the response on a model that is closest to the human scenario, we used human growth plate cartilage specimens. We obtained the human growth plate cartilage from patients undergoing surgery for epiphyseal drilling to restrict the bone growth in patients with leg length differences. The growth plate cartilage biopsies were collected using a biopsy needle (Gallini Biomid, size 7G 10cm, Apgar, Denmark). The obtained biopsies were transferred to a tube containing DMEM-high glucose and 20 µg/ml gentamicin. During culture, medium was supplemented with ascorbic acid (50 µg/ml), beta glycerophosphate (1mM), 0.2% BSA and kept in 37ºC with 5% CO_2 incubator. Following incubation, samples were treated with radial shock waves (500 impulses, 180 mJ, 5 Hz) and cultured for another 48 hours in 37ºC with 5% CO_2 incubator. Samples were washed in 1x PBS and kept at -80°C for RNA isolation and gene expression analysis for chondrogenic genes: *SOX9, GLI1, IHH, COLX, and IGF1* were performed using RT² Profiler PCR Array kit (Qiagen, Germany) **(Figure 7A)**.

4.5 MOUSE ATDC5 CHONDROPROGENITOR CELLS (PAPER IV)

The ATDC5 chondrogenic clonal cell line derived from mouse embryonic carcinoma AT 805 cells were used in this study. Cells at a density of 5000 per cm^2 were plated in a T75 cm^2 flask and cultured in DMEM/F12 containing 5% fetal bovine serum, 1% insulin transferrin selenium, 1% sodium pyruvate and 0.5% gentamicin. At 80% confluence, cells were detached and exposed to 500 impulses, 10 Hz, 180 mJ of radial shock waves in a 1.5 ml Eppendorf tube containing the culture media. A total of 1 million cells were placed per tube. The experiment was repeated thrice with three replicates each. Gene expression was assessed at 0, 30 mins, 1.5, 3 and 14 hours after rSWT using q-PCR. Fold change for Aggrecan (*Acan*), Collagen 2 (*Col2a1*), *Sox9*, alkaline phosphatase (*Alpl*), and *Runx2* gene expression were assessed and normalized to beta-actin (*Actb*, housekeeping gene). The experimental overview is given in **Figure 7B**. Fold change was calculated using 2^-ΔΔCt method.

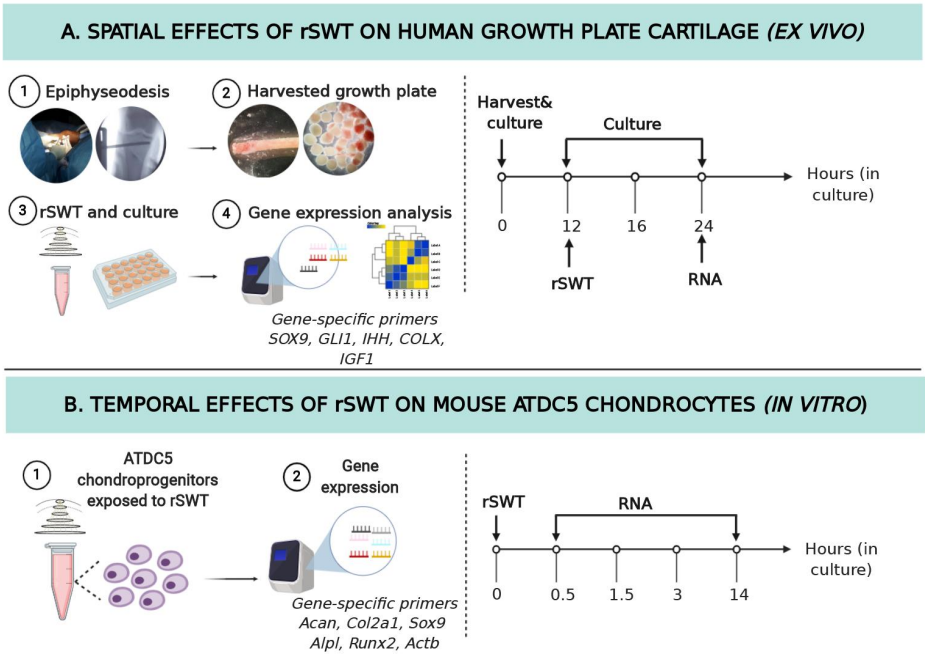

Figure 7. Experimental overview of the *in vitro* studies conducted using A. Spatial effects of rSWT on human growth plate cartilage (1. Procedure to obtain growth plate cartilage, 2. Gross bits of the growth plate cartilage discs, 3. Exposure of growth plate cartilage to rSWT and culture, 4. Gene expression analysis using real-time PCR). **B**. Temporal effects of rSWT on mouse ATDC5 chondroprogenitor cells (1. Exposure of ATDC5 cells to rSWT and 2. Gene expression analysis using real-time PCR).

4.6 STATISTICAL ANALYSIS

All statistical analyses were carried out using GraphPad Prism 8.0 (GraphPad Software, Inc, La Jolla, CA, USA). Data, when normally distributed, were represented as means ± SD for the bone length measurements and growth plate histomorphometric assessments. Kruskal–Wallis and Mann–Whitney U test was used for data that were not normally disturbed **(Paper I, II)**. For comparison between two groups, an appropriate t-test was applied after checking for normal distribution; paired t-test was applied for comparison of final bone length **(Paper III)**. For gene expression analysis (human growth plate study), Mann-Whitney U test was performed, and for ATDC5 study, Student's paired t-test was performed **(Paper IV)**. Probability values < 0.05 indicated a significant difference.

5 RESULTS

5.1 *EX VIVO* AND *IN VIVO* EFFECTS OF RADIAL SHOCK WAVE TREATMENT

We addressed whether radial shock wave treatment improves longitudinal bone growth using two different model systems. An *ex vivo* model of fetal rat metatarsal that is devoid of vascular and growth factors **(Paper I)** and *in vivo* model of healthy New Zealand white rabbits with circulating systemic factors **(Paper III)**. Below is the summary of key findings obtained in the two different models.

5.1.1 Gross

Our proof-of-principle study from fetal rat metatarsal culture experiments demonstrated that a single application of high-energy radial shock wave treatment could stimulate longitudinal bone growth in the absence of any circulating growth factors **(Paper I)**. We then verified this effect *in vivo* by exposing adolescent rabbits to four weekly-sessions of high-energy rSWT. At skeletal maturity, a change in the final bone length was observed **(Paper III)**. Our data, therefore, suggest that radial shock wave treatment contributes to overall increased linear bone growth.

5.1.2 Microscopic

To investigate the microscopic changes in growth plate cartilage, a histomorphometric analysis was performed. Cultured metatarsal bones exposed to high-energy rSWT stained with Alcian blue demonstrated a significant change in the size of the hypertrophic chondrocytes compared to untreated control. This was further supported by an increase in the COLX immunostaining **(paper I)**. In contrast, growth plate sections from rabbits treated *in vivo* with high-energy rSWT revealed no change in chondrocyte hypertrophy, although one animal showed increased bone formation at the site of treatment **(Paper III)**.

5.1.3 Radial shock wave treatment triggers cellular and molecular changes on the growth plate cartilage

To determine the occurrence and location of proliferative cells, metatarsal sections exposed to high-energy rSWT, were stained for PCNA, a marker for proliferating cells. As anticipated, = proliferative zone chondrocytes showed a high prevalence of PCNA positive cells throughout the growth plate compared to untreated control **(Paper I)**. In the rSWT treated rabbit growth plate sections, along with an increased number of proliferative chondrocytes, the number of chondrocyte columns per mm width of the growth plate section was also increased **(Paper III)**. In both the models, we found no change in cell death as determined by TUNEL assay after rSWT treatment. These data indicate that rSWT exerts mitogenic effects in growth plate chondrocytes rather than being detrimental.

We investigated the molecular mechanism behind the observed stimulatory effects of high-energy rSWT treatment by looking for various local growth factors that contribute to long bone growth. We found increased expression of *Pthrp/Gli1* signaling in the rSWT treated metatarsal bones. The expression of *Pthrp* and *Gli1* was localized mainly in the resting and proliferating zone. *BCl2* and *BClx*, the downstream targets of PTHrP, were also increased. Besides, downstream master transcription targets of bone growth, *NF-κB* was also upregulated throughout the growth plate cartilage compared to untreated control. We also found evidence of increased *Igf1* levels, supporting our histomorphometric findings. These observations show that high-energy rSWT can stimulate local upregulation of molecular growth factors responsible for growth plate chondrogenesis. Since the presence of vascularity is a significant factor in endochondral ossification but lacks in the metatarsal organ culture model, we assessed the fetal liver kinase-1 expression, a receptor for vascular endothelial growth factor (VEGF) which was enhanced after high-energy rSWT compared to untreated control. Similarly, rabbits treated with rSWT demonstrated a trend increase in the serum VEGF levels.

Overall, the findings from the *ex vivo* **(Paper I)** and *in vivo* studies **(Paper II)** suggest that rSWT can stimulate longitudinal bone growth and growth plate chondrogenesis under normal physiological conditions **(Figure 8)**.

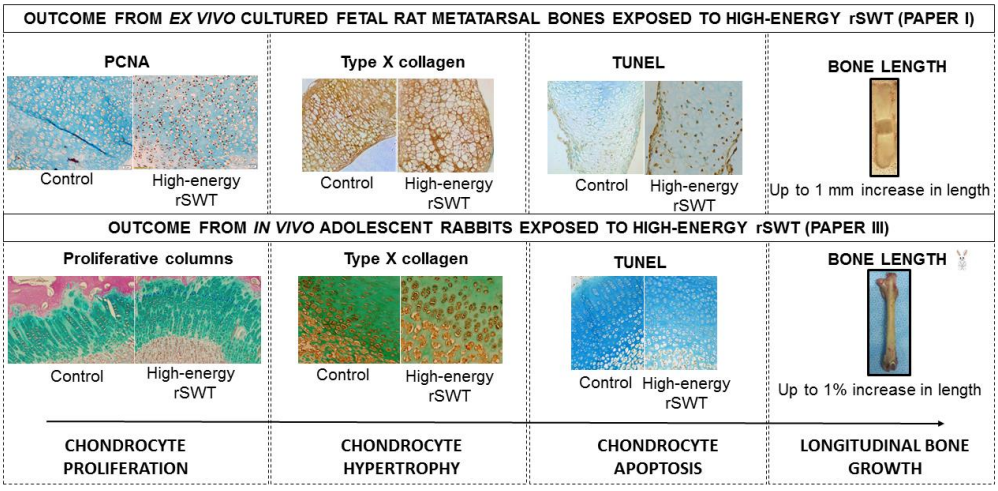

Figure 8. Radial shock wave treatment positively regulates growth plate chondrogenesis in cultured rat metatarsal bones and *in vivo* in rabbits. The progression towards linear bone growth by chondrocyte proliferation, hypertrophy and apoptosis after rSWT in the two models are represented.

5.2 CHONDRO-PROTECTIVE *(EX VIVO)* EFFECTS OF RADIAL SHOCK WAVE TREATMENT UNDER IMPAIRED BONE GROWTH *(*PAPER II*)*

Most of the children present with growth disturbance in the clinics do not have a healthy growth plate. The data from the first set of experiments performed under normal physiological conditions, although provided us with a lead, may not be relevant in this case. To understand if radial shock wave treatment can rescue bone growth in a model of growth failure, we blocked local growth factors. Based on the knowledge we obtained from our previous study, we targeted the hedgehog signaling pathway to induce bone growth impairment in *ex vivo* cultured metatarsal bones **(Paper II)**. Two different small molecule inhibitors that impair hedgehog signaling at different levels were used **(Figure 9)**.

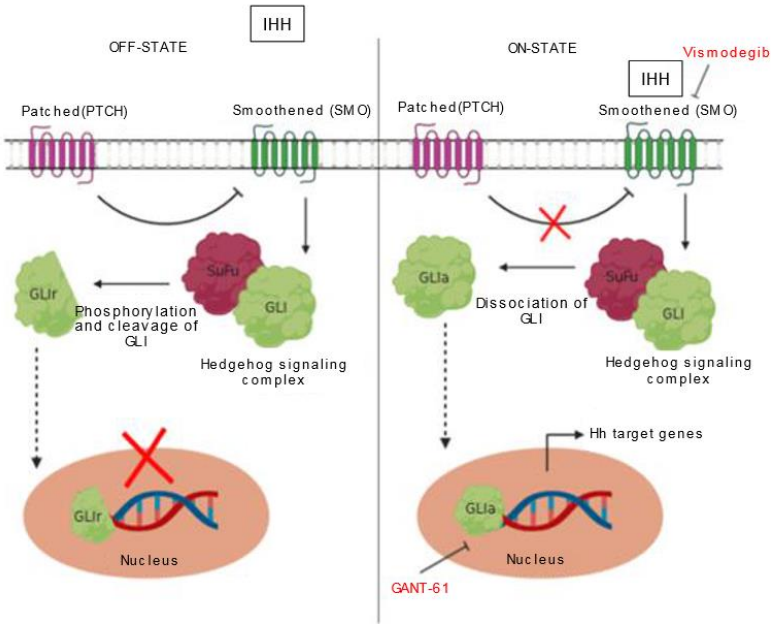

Figure 9. **Canonical activation of the HH/GLI signaling pathway.** In the absence of HH ligand patched (PTCH) inhibits Smoothened (SMO) and the signaling complex SUFU/GLI undergoes phosphorylation to cleave GLI1. The truncated GLI translocates into the nucleus where they inhibit transcription of hedgehog target genes. Upon HH ligand binding (right) the SMO promotes translocation of full-length GLI in active form into the nucleus to activate the transcription of target HH genes. Vismodegib impairs bone growth by blocking SMO (upstream) while GANT61 blocks the transcription of HH target genes (downstream) as depicted in the illustration above.

5.2.1 Rescuing effects of radial shock wave treatment in *ex vivo* cultured metatarsal bones

To understand if the stimulatory effect of shock waves could be achieved in a condition of growth failure, metatarsal bones were exposed to two different Indian hedgehog inhibitors, vismodegib and GLI antagonist (GANT61). Vismodegib binds to Smoothened, a transmembrane protein involved in Hedgehog signal transduction (Gli1/2 inhibited). We specifically chose this small molecule as it is being used in the clinics to treat children with medulloblastoma. During the study period, a paper in Oncotargets was published, which showed that vismodegib caused irreversible growth plate fusion in children as a side effect (Robinson et al., 2017). This motivated us to carry out a proof of principle study in metatarsal bones somewhat to mimic the impaired growth plate chondrogenesis in children. GANT61 on the other inhibits transcription of GLI and acts downstream to Hh signaling. This drug is under preclinical investigation and has not been used to study its effects on longitudinal bone growth.

5.2.2 Bone growth and histomorphometry

To induce transient growth impairment, bones were treated with vismodegib for five days and followed for another ten days in culture. A gradual decline in bone length was observed in the vismodegib treated group compared to untreated control. In contrast, GANT61 was supplemented throughout the culture to recreate an ongoing growth impairment. The decline in growth was observed as early as day 4. A one-time treatment with rSWT rescued the bone growth from both the inhibitors.

When evaluating growth plate morphology, bones treated with vismodegib and GANT61 revealed disrupted columnar organization compared to their respective untreated controls. The ultrastructure of the growth plate was restored and almost near-normal in the bones co-treated with rSWT. A more pronounced reduction in the hypertrophic cell size was observed in both the inhibitor-treated groups, and this was overcome in bones that received rSWT along with the inhibitors. Mechanistic studies for Gli-1 **(Figure 10)** and Ihh revealed a trend increase in the expression of these markers with or without inhibitor treatment.

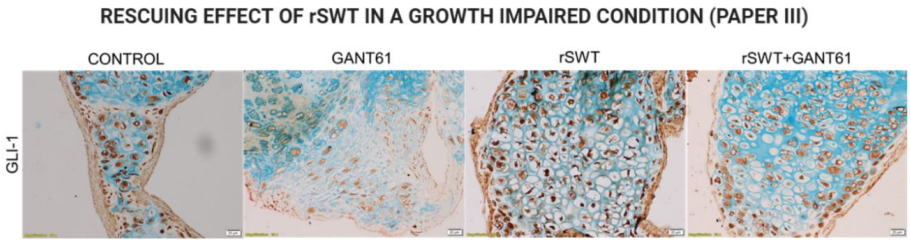

Figure 10. Restoration of GLI1 expression after rSWT. Figures show GLI1 immunostaining in control, GANT61 treated, rSWT treated, and rSWT+ GANT61 treated metatarsal bones. Note the partial restoration of GLI1 expression (brown) in the bones co-treated with rSWT+GANT61 compared to GANT61 alone.

5.3 MOLECULAR MECHANISM OF RADIAL SHOCK WAVE TREATMENT ON HUMAN GROWTH PLATE TISSUE AND MOUSE ATDC5 CHONDROPROGENITORS

To verify our promising preclinical data in cultured fetal rat metatarsal bones and *in vivo* in rabbits, we extended our study to rare samples of human growth plate cartilage cultured *ex vivo* and exposed to rSWT. A pilot study was first conducted to explore the effects of rSWT on chondrogenic markers, and then a more detailed molecular analysis was performed to see how shock waves affect various growth plate zones **(Paper IV)**.

5.3.1 Pilot study - Chondrogenic markers after rSWT

Gene expression analysis for *SOX9* and *COL2A1* was performed at 24-hours after exposure of human growth plate tissues to high-energy rSWT. Since each patient has their basal level expression of gene markers, it was worthwhile to analyze the data by separating them as responders and non-responders to the treatment. We, therefore, decided to individually present the trend increase in gene expression in different patients. An increased expression of *SOX9* and *COL2A1* was found when compared to untreated controls. Three out of the five patient samples showed a mild increase in gene expression, while the other two samples showed a pronounced effect **(Paper III)**.

5.3.2 Spatial regulation of gene expression after rSWT

Based on this lead, we studied the effect of rSWT on spatial gene expression of local molecular factors marking different growth plate zones responsible for proliferation and hypertrophy *SOX9, GLI1, IHH, COLX,* and *IGF1* in five human growth plate tissues. Three different patient samples showed an increase in *SOX9* and *GLI1,* while four showed an increase in *IHH* and *COLX* after treatment. One of the patients showed an increase in *IGF1* levels **(Paper IV)**.

5.3.3 Temporal regulation of gene expression after rSWT

A parallel study was conducted in mouse ATDC5 chondroprogenitor cells to investigate the temporal effects on growth plate chondrogenesis. The expression of *Acan* and *Sox9* showed expression at 3-4 hours after rSWT. The *Col2a1* levels increased at 4 hours showed a sustained expression until 14 hours after the treatment **(Paper IV)**.

6 DISCUSSION

Many of the drugs used in the pediatric population have adverse off-target effects on growth plate chondrocytes, e.g., glucocorticoids (Simon, Fernando, Czernichow, & Prieur, 2002), inflammatory cytokines (Sederquist, Fernandez-Vojvodich, Zaman, & Sävendahl, 2014), and chemotherapy (Eriksson et al., 2012). Several strategies have been investigated in the past for the modulation of bone growth, and some of them are listed in the tables below **(Table 6, 7)**. Herein, our data shows that a single session of rSWT improves longitudinal bone growth in *ex vivo* metatarsal cultures and *in vivo* in rabbits, a consequence linked to augmented proliferation and decreased apoptosis of growth plate chondrocytes. The effect observed *ex vivo* was mediated by upregulation of local growth factors governing longitudinal bone growth. We also show that rSWT rescues the bone length and protects growth plate chondrocytes when there is transient growth failure. Furthermore, the chondrogenic effect of rSWT on human growth plate cartilage was confirmed, where different local molecular factors in each growth plate zone were upregulated. Altogether, the present study focused on delineating how growth plate chondrogenesis and thereby longitudinal bone growth are affected by radial shock wave treatment.

Table 6. Models investigated for growth plate stimulation

Method to create growth stimulation		
Model	**Molecules**	**Reference(s)**
Socs2-/- pups	GH, IGF-1	(Dobie et al., 2015; Mancilla, De Luca, Uyeda, Czerwiec, & Baron, 1998)
E19	IGF-1, PTH	(Coxam, Miller, Bowman, Qi, & Miller, 1995)
E20	Resveratrol	(Karimian et al., 2013)
E20	HNG	(Zaman et al., 2019)
E20	Radial shock wave treatment	(Ramesh, Zaman, et al., 2020)
E20	Lithium Chloride	(Soucek, Zaman, & Sävendahl, 2015)
Method to create growth stimulation		
Model	**Molecules**	**Reference(s)**
Igf1 null mice	Genetic knockout	(J. Wang, Zhou, & Bondy, 1999)
Neonatal Rat bones	Fluoride	(Ma et al., 2019)
E20 bones	FGF2	(Mancilla et al., 1998)
E20 and P8 bones	Dexamethasone	(Chagin, Karimian, Sundström, Eriksson, & Sävendahl, 2010)
E20 bones	IL-1β, TNF-α	(Mårtensson, Chrysis, & Sävendahl, 2004)
E20 bones	Bortezomib	(Eriksson et al., 2012)
E20 bones	Letrozole	(Chagin, Chrysis, Takigawa, Ritzen, & Sävendahl, 2006)
E20 bones	Vismodegib, GANT61	(Ramesh, Sävendahl, Madhuri, & Zaman, 2020)

Longitudinal bone growth depends majorly on the synchronized activities of chondrocytes within the physeal cartilage, and therefore the rate at which proliferation and differentiation occur will reflect in one or more measurable parameters defining chondrogenesis (Emons et al., 2011; E. B. Hunziker, 1994). The metatarsal model used in this study allows for the study of linear bone growth. There is considerable evidence that suggests the influence of biomechanical stimulus in the process of longitudinal bone growth, where more pressure on the growth plate delays bone growth and reduced pressure stimulates growth (Villemure & Stokes, 2009). We also observed that shock wave treatment, a form of mechanotherapy stimulated linear bone growth in *ex vivo* cultured metatarsal bones, suggesting that these bones experienced a reduced pressure (Ramesh, Zaman, et al., 2020). Furthermore, when tested *in vivo* in adolescent rabbits, an increase in tibial length was found. One would postulate that the high-energy dose to be deleterious to chondrocytes, but these findings highlight that a higher dose need not necessarily harm the cells but rather be physiologically beneficial as well. The mechanisms behind the observed stimulation of growth could be envisaged to follow one or a combination of the many proposed sequences.

The cellular response to mechanical signals contributing to the accelerated growth can be evaluated using growth plate histomorphometry. During embryonic development, longitudinal bone growth has three primary components: chondrocyte proliferation, hypertrophy, and cartilage matrix synthesis. Following shock wave treatment, increased cell proliferation was observed in both *ex vivo* cultured metatarsal bones **(Paper I)** and treated rabbit growth plate cartilage **(Paper III)**. Based on these observations, it can be speculated that the stimulation of the resting chondrocytes subsequently amplifies the generation of daughter cells during their recruitment into the proliferative zone, leading to a clonal expansion effect **(Figure 11)** (E. B. Hunziker, 1994). Growth hormone is one such agent capable of producing a differentiation effect while IGF-1 influences the proliferating chondrocytes.

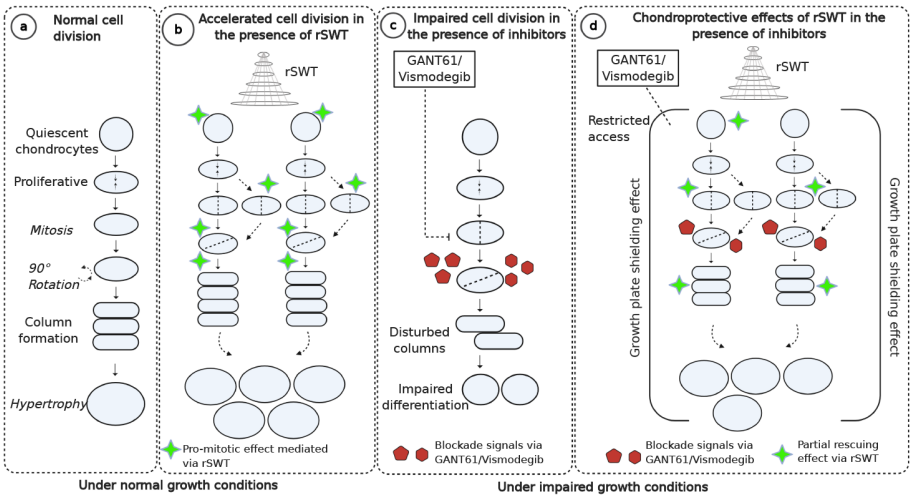

Figure 11. A schematic representation of rSWT mediated effects on chondrocyte proliferation and hypertrophy under normal and growth impaired conditions. A. Normal chondrocyte division **B.** Accelerated cell division after rSWT, **C.** Impaired cell division in the presence of hedgehog inhibitors, **D.** Chondroprotective effect of rSWT in the presence of inhibitors.

Chondrocyte hypertrophy is another essential regulator of linear bone growth (E. Hunziker & Schenk, 1989). Radial shock wave treatment showed a pronounced hypertrophic effect on the metatarsal bones. This could not be verified *in vivo* in rabbits, probably due to their differences in the growth plate mitotic activity across species. Most of the reports advocate that hypertrophic chondrocytes significantly contribute to bone growth under mechanical stimulus, just like during the normal regulation of growth. This indeed is interesting as one would presume that the change in the proliferative columns primarily modulates the growth. However, studies suggest that the hypertrophic zone, being the least rigid region of the growth plate and sensitive to the maximum deformations under mechanical loading, is considered mechanotransduction zone (Villemure & Stokes, 2009). This is probably due to their swollen lacunae. When compression is applied to the growth plate, it reduces the hypertrophic zone thickness and chondrocyte volume with reduced expression of ECM proteins (type II and X). Thus, it is likely that metatarsal growth plate in response to an external mechanical distraction force led to an increase in the increased matrix synthesis, change in tissue and cell morphology, contributing to an overall increase in bone length. Several signaling pathways have been known to play critical roles in influencing growth plate cellular activities (Lui et al., 2010). In this study, we found a causal relationship behind the observed increase in linear bone growth via upregulation of the following local growth factors that are proliferative and/or anti-apoptotic: PTHrP/GLI1, NFkB, IGF1, BCL2, Bcl-X. We speculate that following shock wave treatment there was a direct stimulation of the cell nuclei that are involved in the transcription of genes responsible for chondrocytes proliferation, hypertrophy and apoptosis.

A previous *in vivo* study in rabbits demonstrated an increase in physeal thickness, six weeks after three sessions of lithotripsy with no change in bone length (Ozturk et al., 2008). This is in line with our study, where we also observed a trend of increasing physeal thickness in an immature rabbit model six weeks after rSWT with no effect on limb length. In the subsequent experiments, we used the same methodology and followed up the animals until growth plate fusion and found no change in bone length. We learned that the *in vivo* effects of rSWT are very transient, and a continuous exposure to rSWT would be required for sustained effects on bone growth. We also learned from our in *vivo* experiments that it is difficult to override the intrinsic growth plate memory to alter the bone length by applying a transient treatment. Alternatively, one could postulate that the low-energy dose used in this study could not affect growth and thereby considered a safe dose for non-skeletal disorder and applied around the physis.

Based on these leads, in the next set of experiments, we exposed adolescent rabbits to high-energy rSWT. We then saw an increase in tibial length in these animals. This in line with the only study published so far by Saisu et al., where focal shock wave treatment stimulated final bone length in rabbits (Saisu et al., 2004). From our *in vivo* studies, we learned that systemic factors would try to compensate for minor changes in the bone length, which is easier to observe in species like rodents owing to their increased cellular activity. We did not present any convincing evidence that these models have any relevance to children with growth disturbances. Therefore, we speculated that by creating an adverse environment for the cells to proliferate using an ex vivo metatarsal model, the subsequent effect of rSWT on cell morphology and phenotype would be easier to assess.

Clinical use of vismodegib in children with pediatric brain tumors, has a destructive effect on growth plate chondrocytes (Robinson et al., 2017). To date, no effective treatment is available to protect these bones. An extension study to our preliminary findings, we created a model of impaired bone growth to test if shock wave treatment could reverse these detrimental effects. To understand the physiological role of rSWT during impaired growth plate chondrogenesis, we blocked the hedgehog signaling pathway that has a role in chondrocytes proliferation and differentiation using two antagonists, vismodegib and GANT61. The inhibitors disturbed chondrocyte proliferation and hypertrophic differentiation, leading to a decreased bone length **(Paper II)**. These data are in line with the recent clinical observation where vismodegib has been reported to cause permanent growth plate fusion in children. While when treated with rSWT, we found that despite the presence of the inhibitors, priming the bones with rSWT stimulated an intrinsic mechanism to partially protect the cells from the damage; resulting in rescued longitudinal growth of fetal rat metatarsal bones (Ramesh, Sävendahl, et al., 2020).

The exact mechanism underlying the chondroprotective effect of rSWT remains unclear and requires further investigations. It is indeed interesting to study how mechanical waves can modulate the molecular activity in growth plate chondrocytes and have long-term effects on chondrogenesis and longitudinal bone growth. The mechanisms through which these local signals are translated into gene expression have never been investigated in the human growth plate. Therefore, we corroborated our findings in cultured human growth plate tissue from prepubertal children and found increased expression of genes associated with chondrogenesis further strengthening our preclinical findings. To our knowledge, we are the first to demonstrate the spatial effects of rSWT on different growth plate zones in an *ex vivo* model of cultured human growth plate cartilage where different genes where chondrogenic genes were enhanced in different patients. Besides, we also explored the temporal regulation of rSWT using mouse ATDC5 chondroprogenitor cells where genes responsible for matrix synthesis were upregulated **(Paper IV)**. These findings supported that there is increased matrix production following mechanotherapy.

6.1 LIMITATIONS

This study has several limitations, and the reader should interpret our main findings in light of each limitation Firstly, the *ex vivo* model of cultured metatarsals allowed us to investigate the local effects but not the endochondral ossification as it lacks vascularity **(Paper I, II)**. Secondly, we did not test the efficacy of multiple sessions of rSWT on growth plate chondrogenesis. This would have given us more information on when to administer the subsequent shock wave treatment. Thirdly, we used very few patient samples in the human growth plate experiments, and each patient had a different response in gene expression pattern **(Paper IV)**. This could be due to the inherent biological heterogeneity. Finally, the effect of rSWT on primary cilia was not characterized in this study. This would have given us more insight into the mechanotransduction effect of rSWT on longitudinal bone growth.

7 CONCLUSIONS

Our findings suggest that radial shock wave treatment positively regulates longitudinal bone growth:

1) Radial shock wave treatment enhances chondrocyte proliferation and hypertrophy by increasing the NFkB/Gli1 signaling pathways in cultured rat metatarsal bones;

2) Radial shock wave treatment stimulates growth plate chondrogenesis and longitudinal bone growth in young and adolescent rabbits;

3) Radial shock wave treatment protects bone growth and growth plate chondrocytes under conditions of impaired bone growth involving suppressed Hh signaling;

4) Radial shock wave treatment upregulates genes modulating distinct zones of proliferation and hypertrophy in cultured human growth plate cartilage

These findings provide new knowledge and opportunities for a better understanding of the molecular and cellular mechanisms by which mechanotherapy aids in accelerating bone growth and growth plate chondrogenesis.

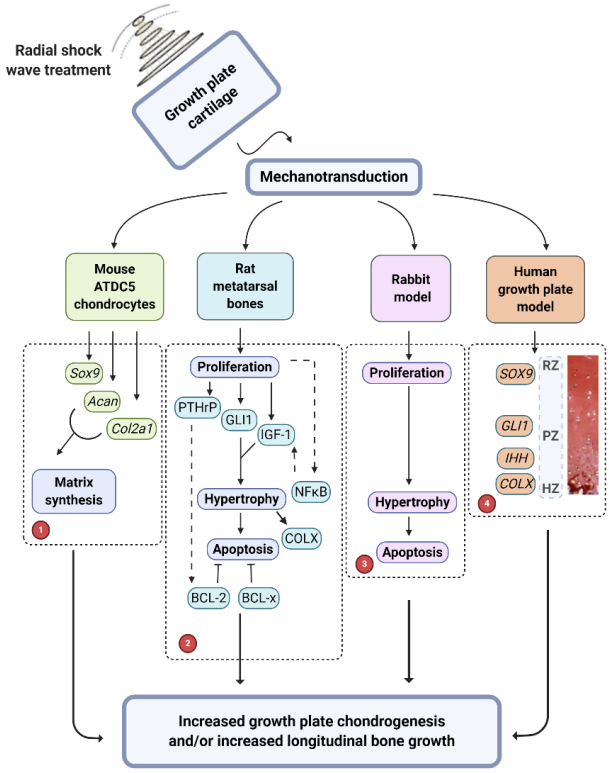

Figure 12. A schematic summary of data obtained in 1. Mouse chondrocytes, **2.** *Ex vivo* cultured rat metatarsal bones, **3.** *In vivo* in rabbits, and **4.** *Ex vivo* cultured human growth plate cartilage. Molecular markers increased after rSWT are detailed in the diagram.

8 POINTS OF PERSPECTIVE

Over the last few decades, our understanding of the structural, cellular, paracrine and endocrine regulation of longitudinal bone growth at the growth plate level has substantially advanced. In this thesis, I have focused on recent advances in the understanding of growth plate biology and treatment of growth failure.

The findings from the current thesis have identified important biological mechanisms and many molecular targets regulating longitudinal bone growth following radial shock wave treatment. These may eventually lead to a promising potential new treatment for growth failure that is currently being evaluated in preclinical studies. However, like every research, our study yields more questions than it answers.

For example, i) can radial shock wave treatment rescue impaired bone growth *in vivo*?; ii) which intrinsic mechanotransduction signals are activated?; iii) what happens when continuously exposing to shock wave treatment in the presence of systemic hormones and circulating growth factors, will it be synergistic or will they nullify each other?

In the future, regulating the signaling cascades by which shock waves promote linear bone growth may develop as a new evolving strategy for growth plate deformities, and I am happy that the findings from this thesis would have laid the foundation for future research in growth plate mechanobiology.

9 ACKNOWLEDGEMENTS

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It is often not easy to have the best of both worlds. I take this opportunity to express my gratitude to all those for having brought out the best in me.

During the initial period of a scientific career, we often get influenced by our supervisor. In my case, I had the fantastic opportunity to be supervised by two clinician-scientists (**Profs**. **Lars Sävendahl and Vrisha Madhuri)** who are the best in their fields, intensely focused, hardworking, have competitive spirit and real fighters! I hope I will make both of you proud someday! Thank you both for your efforts in shaping my thoughts.

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Esther_histo
Suhasini
Agnes
Sevanthy
Ahmed_Reda
Martin_Ritzén
Vicky_Basky
Klas_Blomgren
Muthu_anna
Lalit_Kumar
Iulia_Savchuk
Leif_Karlsson
Dolly_Daniel
Ola_Nilsson
Ann_David
Svetlana_Lajic
Chinmaya_Vidayala
Cecilia_Götherström
Vastchala
Jan-bernd_stukenborg
Pavithra_animalhouse
Lilly_Velentza
Suresh_Devasahayam
Farasat_Zaman
Saritha
Geetha
Andrei_Chagin
Devendran
CMC
Dasarthi
Jerry_Japan
Kai_Zhou
Tamilvannan
Ahmed_Osman
Therese_Cedervall
Vikram_Mathews
Michaele_Liverts
Ramesh_animalhouse
Ondrej_Soucek
Alok_Srivastava
Arun_John_Paul
Archana_Kini
Shanthini
Maryam_Irvani
Sam_Vijay
Lavanya
Camilla_Adle
Olle_Söder
Grandma
Ashok_histo
SASTRA
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Zhengpei_Zhang
Esther_POR
Aneesha
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Caroline_Rådestad
CSCR_students
Eva_Pontén
Anupama
Susanne_Hallberg
Lars_Sävendahl
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Deutsche
Abdul
Jennifer_Frithiof
Renita_Raymond
Abu_Faroq
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Valentina_Pampanini
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Salar_Abbas
Franklin
TT_Aravind
Annika_Goos
Bettina_Celvin
Krtistina_Tammimies
CSCR_housekeeping
Abel
Yunhan_Zhao
Karthikeyan
Kaushik
Farhan
Anna_Sandberg
Sakthi_Ponandai_Srinivasan
Arun_animalhouse
Christine_Carlsson_Skwirut
Cousins
Vignesh_Kumar_Rangasami
Magdalena_Kurek
Vrisha_Madhuri
Nageswara_Boggavarapu
Venkatesh
Sundu
Alfred_Job_Daniel
Nithin_Sam
Sasank
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Lillian_Jallow_Walther
Bibhu_Dutta_Sahoo
Indian_Street_food_Stockholm
Andrea_Merker
Cecilia_Dominguez
Valerria_Messina
Konstantin_Svechnikov
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